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Cell-Bound Thiaminase I of *Bacillus thiaminolyticus*

C. COE AGEE[1], JUDY H. WILKINS[2], AND R. L. AIRTH[3]
Cell Research Institute, University of Texas, Austin, Texas 78712

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The distribution of the extracellular enzyme, thiaminase I, was determined for logarithmically growing cultures of *Bacillus thiaminolyticus*. About 60% of the enzyme is associated with the cells throughout the growth cycle. The remainder of the enzyme is in the culture medium. The release of the cell-bound thiaminase I is examined under a variety of conditions. The rate and extent of release is dependent on the pH and the nature of the incubation solution. The release process appears to be relatively independent of de novo protein synthesis, energy derived from oxidative phosphorylation, or divalent metal ions. The absence of carbon or nitrogen sources has little effect on the release of the enzyme. Cell-bound thiaminase I probably is the immediate precursor for extracellular thiaminase I found in the culture medium. Washed cells continue to release thiaminase I at the expense of cell-bound enzyme. In addition, purified cell-bound thiaminase I is indistinguishable from purified extracellular thiaminase I by a number of physical and kinetic criteria.

Various laboratories have examined the sites of synthesis and mechanisms of release of bacterial extracellular enzymes (4). Thiaminase I (EC 2.5.1.2), an extracellular enzyme produced in large quantities by *Bacillus thiaminolyticus*, provides an interesting system for the study of this problem. This enzyme catalyzes a base-exchange reaction which results in the destruction of thiamine (Fig. 1). Destruction of this vital cofactor inside the cell would disrupt cellular metabolism. It is therefore likely that thiaminase I is synthesized either as an inactive precursor (eg., zymogen) or within some type of cellular compartment.

Preliminary experiments with antibody to extracellular thiaminase I indicate the presence of an enzymatically inactive thiaminase I in the debris of sonically disrupted *B. thiaminolyticus* cells (11). This observation therefore supports the zymogen theory of thiaminase I origin. Our attempts to isolate this material lead to the discovery of a previously unreported active cell-bound thiaminase I.

This communication reports the conditions which favor the production of active, cell-bound thiaminase I and methods which release this enzyme. Purification and partial characterization of cell-bound thiaminase I suggests that the cell-bound enzyme is the precursor for the extracellular thiaminase I.

[1] Present address: Department of Biochemistry, University of Illinois, Urbana, Illinois 61801.

[2] Present address: Department of Biochemistry, Virginia Polytechnic Institute, and State University, Blacksburg, Virginia 24060.

[3] Deceased.

MATERIALS AND METHODS

Culturing of the organism. Cultures of *B. thiaminolyticus*, strain M, were grown in a medium consisting of 30 mM sucrose, 17 mM sodium citrate, 8 mM KH_2PO_4, 42 mM Na_2HPO_4, 50 mM $(NH_4)_2HPO_4$, 0.1 mM $FeSO_4 \cdot 7H_2O$, 0.05 mM $MgSO_4 \cdot 7H_2O$, and 3×10^{-8} M thiamine·hydrochloride. The medium (without thiamine or magnesium sulfate) was adjusted to pH 7.8 prior to autoclaving. Thiamine and magnesium sulfate were sterilized by filtration (0.22 μm pore size; Millipore Corp.) and added aseptically to the cooled medium. Cultures were grown at 37 C with shaking and then harvested by centrifugation (0-2 C) at 10,000 $\times$ *g* for 20 min.

Release of cell-bound thiaminase I. The cell pellets from two or three, 500-ml, 20-h cultures were washed once with 20 ml of ice-cold, 0.85% sodium chloride solution usually containing 100 μg of chloramphenicol (CAM) per ml. About 10 to 20% of the total cell-bound thiaminase I activity was removed by this wash. Little additional activity (<5%) was removed by repeated washing of the cells under these conditions. To determine the effects of various environments on the release of the cell-bound enzyme, cells were incubated in various test solutions consisting of buffers, salts, sugars, media, or deionized water. Washed cells were resuspended at approximately 1/100

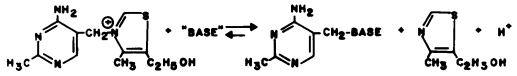

FIG. 1. *Generalized base-exchange reaction catalyzed by thiaminase I.*

of their original culture volume in ice-cold, deionized water containing 200 μg of CAM per ml. Immediately, a volume of this cell suspension was added to an equal volume of a test solution. The test solution and cell suspension were mixed; they were incubated in a water bath. The final volumes of the incubation mixtures varied from 6 to 30 ml depending on the experiment. At the end of the incubation period the cells were removed by centrifugation for 1 min at 30,000 × *g*. The supernatant fluids and cell pellets were then assayed for thiaminase I activity and protein concentration.

Purification of cell-bound thiaminase I. Preparation of crude enzyme. Six liters of culture grown for 24 h on defined medium were harvested (0–2 C) by centrifugation at 10,000 × *g*. The pelleted cells were resuspended in 30 ml of 0.05 M sodium citrate buffer at pH 4.7 and incubated for 15 to 20 min at 37 C. The cells were removed by centrifugation at 30,000 × *g* for 30 min at 0 to 2 C. The supernatant solution containing the thiaminase I activity was decanted into a Diaflo ultrafiltration cell equipped with a UM-10 membrane (Amicon Corp., Lexington, Mass.), dithiothreitol (DTT) was added to a final concentration of 10^{-3} M, and the supernatant solution was concentrated (0–2 C) to 3 or 4 ml under nitrogen gas at 50 psi.

Gel filtration. The concentrated supernatant solution was centrifuged (30,000 × *g* for 30 min) to remove precipitated proteins. The 30,000 × *g* supernatant solution was loaded on a Sephadex G-150 column (2.5 by 36 cm) equilibrated in 0.1 M sodium phosphate buffer at pH 7.0 and eluted at 0 to 2 C with the same buffer at a flow rate of about 10 ml per h. Three-milliliter fractions were collected and assayed for thiaminase I activity. Fractions containing thiaminase I activity were pooled, DTT was added to a final concentration of 10^{-3} M, and the enzyme solution was concentrated as before.

Preparative gel electrophoresis. A prep-disk polyacrylamide electrophoresis column (PD-150, Canal Industrial Corp., Rockville, Md.) was prepared containing a column (2.5 to 3.0 cm) of a 10% cross-linked, pH 8.5 tris(hydroxymethyl)aminomethane (Tris)-glycine separating gel. The separating gel contained 10^{-4} M DTT. The one-centimeter long, 3% stacking gel (pH 6.9) was polymerized with ammonium persulfate instead of riboflavin. The electrode buffers were 0.03 M Tris-glycine at pH 8.0. The elution buffer was a pH 9.0, 0.004 M Tris-glycine buffer containing 10^{-4} M DTT. The concentrated enzyme solution from the Sephadex column was adjusted to pH 8 with 1 M Tris and then loaded on the prep-disk column. Electrophoresis was performed at a current of four milliamps and 4 C. The proteins were eluted from the column at a buffer flow rate of 20 to 30 ml per h and collected as 3-ml fractions. Fractions containing thiaminase I activity were pooled and dialyzed against several changes of 0.01 M sodium phosphate buffer, pH 7.0, and then stored at 0 to 4 C. Table 1 presents the data from a typical purification experiment.

Cell breakage. Cell pellets were resuspended in 0.1 M sodium citrate at pH 3.0 or 4.7 and subjected to ultrasonic oscillation by using a Branson model S-75 sonifer equipped with a microprobe and operating at 10,000 cycles/s for a total time of 30 s (six 5-s bursts with 10- to 15-s cooling periods between bursts). Cooling was accomplished with an ice, water, sodium chloride bath. Cellular debris were removed by centrifugation at 30,000 × *g* for 30 min. This procedure does not result in the denaturation of thiaminase I.

Enzyme assays. Thiaminase I activity was measured by either of two methods. In most cases, the spectrophotometric assay of Douthit and Airth (2) as modified by Wittliff and Airth (13) was used. Certain specific instances employ a radiometric assay in which the disappearance of thiazole-2-^{14}C-thiamine·hydrochloride (25.2 mCi/mmol) and the appearance of thiazole-2-^{14}C was monitored. Reactions contained 1.15 mM aniline, 0.1 M sodium phosphate buffer, pH 5.8, and 39.6 μM thiazole-2-^{14}C-thiamine·hydrochloride in a total volume of 0.1 ml. Reactions were prepared and run as described for the spectrophotometric assay. Reactions were stopped by the addition of an equal volume of saturated trichloroacetic acid solution and the reactants and products were then separated on silica gel G thin-layer chromatographic plates developed with 1-butanol-acetic acid-water (4/1/5). The dried plates were cut at 1-cm intervals and the powder was suspended in Bray's solution and counted in a liquid scintillation counter. Heated enzyme (100 C for 30 min) and controls lacking enzyme were also analyzed. One unit of thiaminase I activity was defined as the amount of enzyme required to catalyze the formation of 1 μmol of product in 1 min at 25 C.

Isocitrate dehydrogenase (EC 1.1.1.41) activity is determined by the method of Ochoa (7). Cells for this assay are sonically disrupted in 1 mM Tris-hydrochloride buffer, pH 8.0. One unit of ICDH activity is defined as the amount of enzyme which catalyzes the reduction of 1 mol of nicotinamide adenine dinucleotide (NADP) to $NADPH_2$ in 1 min at 37 C.

Protein was estimated by the method of Lowry et

TABLE 1. *Purification of thiaminase I*

Step	Vol (ml)	Protein (μg/ml)	Activity (mU/ml)	Total activity (mU)	Specific activity (mU/mg)
Release supernatant[a]	32	1,270	970	30,845	765
G-150 pool[b]	55	112	393	21,615	3,500
PD-150 pool[c]	65	29	177	11,505	6,100

[a] pH 4.7 sodium citrate, 30,000 × *g* incubation supernatant fluid.

[b] Pooled activity fractions from Sephadex G-150 column.

[c] Pooled activity fractions from P-150 prep-disk electrophoresis column.

al. (5) by using bovine serum albumin as a standard. The methods of Shapiro et al. (10) and Ornstein (8) were employed for sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis and analytical polyacrylamide disk-gel electrophoresis, respectively. Immunodiffusion was performed by the double-diffusion method described by Ouchterlony (9).

Chemicals and organism. Chloramphenicol was a gift of H. E. Mechame of Parke, Davis and Co. Thiazole-2-^{14}C-thiamine·hydrochloride was purchased from Amersham/Searle. *B. thiaminolyticus* strain M was originally supplied to us by R. Hayashi of the Department of Microbiology, Yamaguchi University School of Medicine, Ube, Japan. Rabbit antibodies against purified extracellular thiaminase I were prepared by J. L. Wittliff (14).

RESULTS

Douthit and Airth (2) first investigated the presence of a cell-bound thiaminase I in *B. thiaminolyticus*. They reported that throughout the growth cycle less than 10% of the thiaminase I activity was cell-bound and greater than 90% of the enzyme occurred in the culture medium. These initial studies involved suspension of cells in distilled water and complete cellular disruption by sonic oscillation. Subsequent studies by Wang et al. (12) and Agee and Airth (1) suggest that these initial studies present a false picture of the distribution of thiaminase I. Specifically, Agee and Airth (1) have shown that under certain conditions both cell-bound and extracellular thiaminase I are rapidly inactivated in the presence of equimolar quantities of thiamine. Wang et al. (12) reported that complete sonic disruption of *B. thiaminolyticus* released about 3×10^{-20} mol of thiamine and thiamine phosphates per cell. Calculations based on the data presented here (Fig. 2) show that cultured under optimal conditions *B. thiaminolyticus* has about 2.5×10^{-20} mol of cell-bound thiaminase I per cell. Thus, much of the cell-bound enzyme may have been inactivated by the intracellular thiamine and thiamine phosphates released during sonic treatment. In addition, the sonic treatment procedure used in the earlier studies was too harsh, resulting in some sonic denaturation of thiaminase I. Full power, continuous sonic treatment of a cell-free extract of thiaminase I results in the loss of as much as 30% of the enzyme's activity in 60 s. It therefore seemed possible that much of the cell-bound enzyme was inactivated during the isolation procedure used in the earlier studies. We, therefore, reexamined the distribution of thiaminase I activity in actively growing cultures of *B. thiaminolyticus*.

If cells are disrupted in pH 3.0 citrate prior to assay, approximately 60% of the total thiaminase I activity resides in the cellular fraction throughout the logarithmic phase of growth (Fig. 2). The observed increase in thiaminase I activity per cell is consistent with the data reported earlier for the extracellular enzyme (2). The extracellular enzyme apparently does not arise from cell lysis since isocitrate dehydrogenase (ICDH), a known intracellular enzyme (7), is found exclusively inside the cell at all stages of growth.

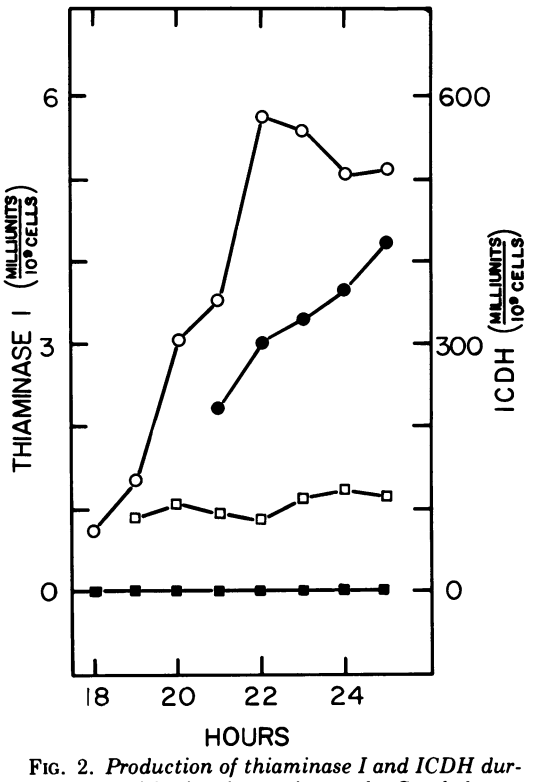

FIG. 2. *Production of thiaminase I and ICDH during the logarithmic phase of growth. Symbols:* ○, *cell-bound thiaminase I;* ●, *extracellular thiaminase I;* □, *intracellular ICDH;* ■, *extracellular ICDH.*

Further proof that the thiaminase I activity found with the cellular fractions is outside the cells' permeability barrier but still firmly attached to the cell comes from experiments in which cells were incubated with antibody to purified thiamine I. Saline-washed cells were rapidly agglutinated in 0.85% NaCl in the presence of antithiaminase I antibody. However, after the removal of thiaminase I by incubation for 15 min at 37 C in 0.05 M sodium citrate buffer (pH 4.7) the cells were not agglutinated by the antibody. Cells incubated for 15 min in sodium citrate, pH 4.7 at 0 C, a temperature at which less than half of the enzyme is released from the cell, still agglutinated in the presence

of the antibody. Thus cell-bound thiaminase I must be at or near the cells' surface to be accessible to the antibody and the enzyme must be rather tightly bound to the cell or cell agglutination would not have occurred.

These data suggest two possible relationships between cell-bound and extracellular thiaminase I. First, cell-bound and extracellular thiaminase I can be two separate enzymes with similar catalytic properties but different functional roles. Secondly, the cell-bound enzyme may be the immediate precursor to the extracellular enzyme.

Two approaches can be used to decide between these alternatives. First, one can examine the conditions which affect the release of the cell-bound enzyme. Second, one can purify the cell-bound enzyme to apparent homogenity and compare its physical and catalytic properties with the extracellular enzyme.

Table 2 shows that saline-washed cells continue to release thiaminase I when suspended in fresh medium. Cell-bound thiaminase I decreases as extracellular thiaminase I increases during the incubation. Thus, the cell-bound enzyme provides an apparent source for extracellular thiaminase I. Protein synthesis and oxidative phosphorylation inhibitors, or the absence of carbon sources, nitrogen sources, or divalent cations do not affect the release of the enzyme from the cells.

Examination of the release phenomenon through longer term incubations is impractical for under these favorable conditions cell division interferes with these assays. Similarly, long-term assays with CAM, dinitrophenol (DNP), or ethylenediaminetetraacetate (EDTA) are also impractical due to cell death. We therefore sought alternate methods for studying the release of the enzyme. Accordingly, we observed the release of the enzyme from cells suspended at much higher cell densities and incubated in various solutions under a variety of conditions.

Figure 3 and Table 3 indicate that the release of cell-bound thiaminase I is influenced by the composition and pH of the incubation solution. In general, acidic pHs (4.7) or hypotonic conditions (deionized water) favor release of the enzyme. Further, the increased release of the enzyme during incubations with acidic buffers (ie., near pH 4) is not reflected in increased release of protein at these acid pHs. Thus, the enzyme released at pH 4 to 6 has a higher specific activity than the thiaminase I released at pH values of 6 and above or 4 and below.

TABLE 2. *Release of cell-bound thiaminase I from washed cells*[a]

Incubation medium	Thiaminase I activity (mU/ml)	
	Cell-bound	Released
Complete (minus thiamine-hydrochloride)[b]		
Zero time	53	28
30 Min-incubation at 37 C	9	86
– Carbon source	11	105
– Nitrogen source	12	89
– Carbon and nitrogen sources	13	116
+ 100 μg of CAM per ml	12	109
+ 10^{-4} M DNP	9	103
+ 10^{-2} M EDTA, – Fe^{2+} and Mg^{2+}	8	110

[a] Cells were washed with saline solution without CAM.

[b] Defined medium as described in Materials and Methods.

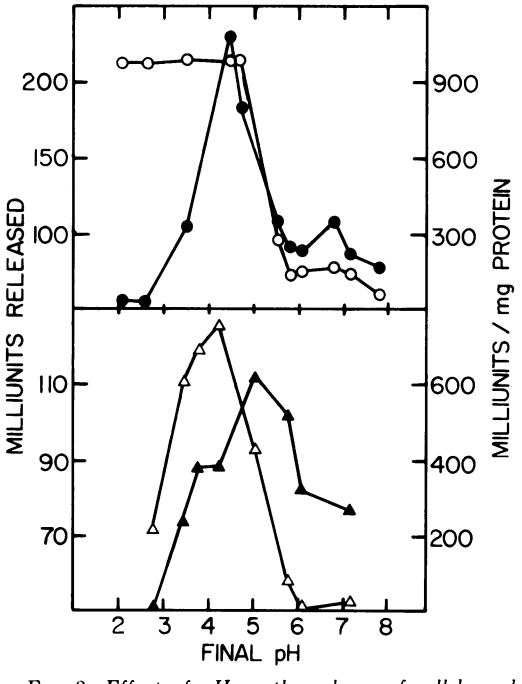

FIG. 3. *Effect of pH on the release of cell-bound thiaminase I. Saline-washed, CAM-treated cells are incubated for 5 min at 37 C in 0.1 M sodium phosphate buffers. Symbols: ○, thiaminase I activity released; ●, specific activity of thiaminase I released; or 0.1 M sodium citrate buffers, △, thiaminase I activity released; ▲, specific activity of thiaminase I released. The pH of the medium is measured at the end of the incubation period after the removal of the cells by centrifugation.*

TABLE 3. *Release of cell-bound thiaminase I in various solutions*

Incubation solution	mU	Activity released during incubation[a] (% of total)	mU	Activity removed by sonic treatment[b] (% of total)
0.05 M sodium citrate, pH 4.7	2,475	92	208	8
0.05 M sodium citrate, pH 5.9	1,206	41	1,705	59
Deionized water[c]	2,454	89	291	11
0.15 M mannitol[c]	894	35	1,674	65
0.075 M sodium chloride[c]	645	27	1,716	63
0.137 M (0.85%) sodium chloride[c]	811	30	1,914	70
0.15 M glucose[c]	1,071	38	1,706	62

[a] Saline-washed, CAM-treated cells were suspended in the solutions, incubated for 5 min at 37 C, centrifuged, and the supernatant fluids were assayed for thiaminase I activity.

[b] At the end of the incubation period, the pelleted cells were sonically treated at pH 4.7 (see Materials and Methods) and the supernatant fluids were assayed for thiaminase I activity.

[c] Unbuffered solutions were adjusted to pH 7 with sodium hydroxide prior to the addition of cells.

More importantly, other experiments show that the enzyme does not return to the cell upon raising the pH or adding an osmotic support to the suspension (Tables 4 and 5).

The release of cell-bound thiaminase I in response to the pH of the incubation medium is not the result of extensive cell lysis. Microscope examination of the cells before and after incubation does not reveal gross changes in their morphology. In addition, viable cell counts on brain-heart-infusion agar plates indicate that 90% of the cells survive 5-min incubations in 0.05 M sodium citrate at pH values of 3.8 and above. Below pH 3.8 the survival rate decreases rapidly. These observations do not reflect a pH-mediated activation or inactivation of the enzyme. The activity of a crude, cell-free thiaminase I solution is stable for up to 18 h at room temperature at pH values from 2 to 7.

It is probable that the varied extents of thiaminase I release shown in Table 3 and Fig. 3 reflect differences in rates of thiaminase I release rather than the total amount of enzyme releasable. Accordingly, we examined the release of cell-bound thiaminase I as a function of the length of the incubation period (Fig. 4). At zero time, the amount of thiaminase I activity released is a function of the pH of the incubation medium. However, the subsequent rate and extent of release of the enzyme is influenced by both the pH and the nature of the incubation medium.

These results cannot be due to differential rates of thiaminase I synthesis during the incubations. In all cases the total amount of thiaminase I activity remained constant throughout the incubation period. In addition, the release of the enzyme from cells suspended in sodium citrate at pH 4.7 is not affected by the addition of inhibitory levels of known protein synthesis, transcription, or oxidative phosphorlation inhibitors (Table 6). Similar results were obtained at pH 5.9.

Thus, greater than 90% of cell-bound thiaminase I is available for release. Further, with the conditions for the release of the cell-bound enzyme firmly documented, a comparison of extracellular and cell-bound thiaminase I is possible.

The specific procedures that purify extracellular thiaminase I (3, 6, 13) do not yield similarly homogeneous thiaminase I preparations when applied to cell-bound enzyme. We therefore devised a purification procedure for cell-bound thiaminase I which yields a single,

TABLE 4. *Release of cell-bound thiaminase I*

Incubation solution[a]	Thiaminase I activity (mU)	
	Cell-bound (released by sonic treatment)	Extracellular (released during incubation)
Sodium citrate-phosphate, pH 4.7[b]		
Zero time	508	725
15-Min incubation	15	1,242
Adjusted pH to 7.0 with NaOH		
Zero time after pH adjustment	37	1,198
15 Min after pH adjustment	43	1,221
Deionized water, pH 7.0		
Zero time	1,152	88
15-Min incubation	246	1,006
Addition NaCl to final concn. of 0.85%		
Zero time after NaCl addition	196	1,029
15 Min after NaCl addition	185	1,047

[a] Saline-washed, CAM-treated cells were suspended and incubated at 37 C. At the indicated times samples were withdrawn, centrifuged, and the supernatant fluids and cells were assayed for thiaminase I activity.

[b] Citric acid (0.05 M) and 0.1 M Na_2HPO_4 were mixed to a final pH of 4.7.

TABLE 5.

Cells	Thiaminase I activity (mU)	
	Cellular fraction (released by sonic treatment)	Supernatant fraction[a] (extracellular)
Released cells[b]		
Zero time	30	0
30-Min incubation	9	0
Released cells + crude thiaminase I		
Zero time	352	2,374
15-Min incubation	431	2,378
30-Min incubation	442	2,367

[a] The supernatant fluid containing the thiaminase I activity from the pH 4.7 incubation was dialyzed into 0.1 M sodium phosphate, pH 7.0, and then concentrated fourfold in a Diaflo ultrafiltration apparatus. Precipitated proteins were removed by centrifugation. The "released cells" were resuspended and incubated in either the concentrated enzyme solution or 0.1 M sodium phosphate buffer, pH 7.0. Samples were removed at the indicated times, centrifuged, and the supernatant fluids and cells were assayed for thiaminase I activity.

[b] Saline-washed, CAM-treated cells were freed of cell-bound thiaminase I activity by a 15-min incubation at 37 C in 0.05 M sodium citrate, pH 4.7, followed by three washes with ice-cold 0.85% sodium chloride solution.

homogeneous protein on gel electrophoresis in Tris-glycine buffer or buffered SDS solution. This same "homogeneous" cell-bound thiaminase I does reveal slight inhomogenity upon ultracentrifugal analysis, but this may also represent polyacrylamide contamination from the final step of the purification procedure.

This purification of cell-bound thiaminase I, and the availability of previously purified extracellular thiaminase I allow one to ask if cell-bound and extracellular thiaminase I are the same protein. Mixtures of purified cell-bound and extracellular thiaminase I migrate as a single band in pH 8.9 analytical polyacrylamide electrophoresis gels. Antibody to purified extracellular enzyme gives only one precipitin band in double-diffusion-agar gels when reacted with cell-bound or extracellular thiaminase I either separately or mixed together (Fig. 5). The observed ultraviolet light spectrum of the purified cell-bound enzyme and that previously reported for extracellular thiaminase I (13) are similar with absorption maxima and minima at 277 and 252 nm, respectively, and optical density at 280 nm (OD_{280nm})/OD_{260nm} ratios of

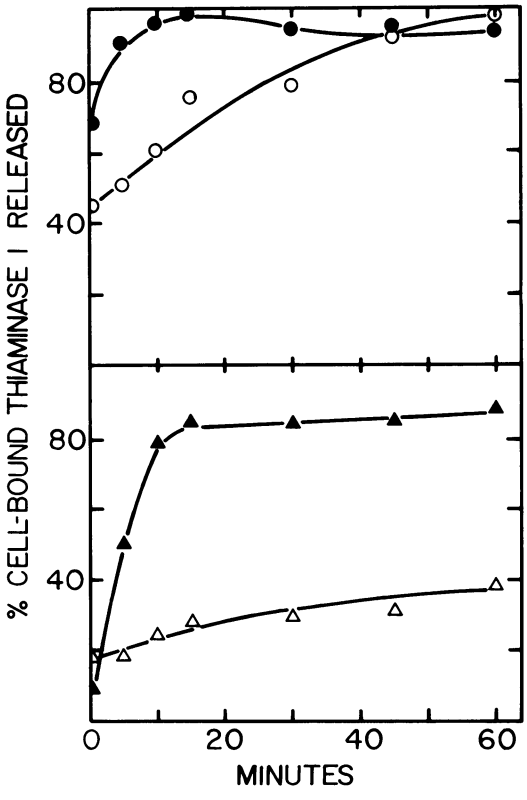

FIG. 4. *Release of cell-bound thiaminase I as a function of the incubation time. Saline-washed, CAM-treated cells are suspended in various solutions and incubated at 37 C. At each time interval, a sample is removed, centrifuged, and the cells and supernatant fluid were assayed for thiaminase I activity. Symbols:* ●, *0.05 M sodium citrate, pH 4.7;* ○, *pH 5.9;* ▲, *deionized water;* △, *0.15 M mannitol.*

TABLE 6. *Effects of inhibitors on the release of thiaminase I from saline washed cells*[a]

Incubation solution	Thiaminase I activity released (mU)
0.05 M sodium citrate, pH 4.7, zero time	98
0.05 M sodium citrate, pH 4.7, 30 min	184
+ 10^{-4} M DNP	168
+ 10^{-3} M puromycin	190
+ 10^{-5} M actinomycin D	174
+ 10^{-3} M CAM	179

[a] Cells were washed, resuspended, and incubated (at 37 C) as described in the Materials and Methods except that the wash and resuspension solutions did not contain CAM.

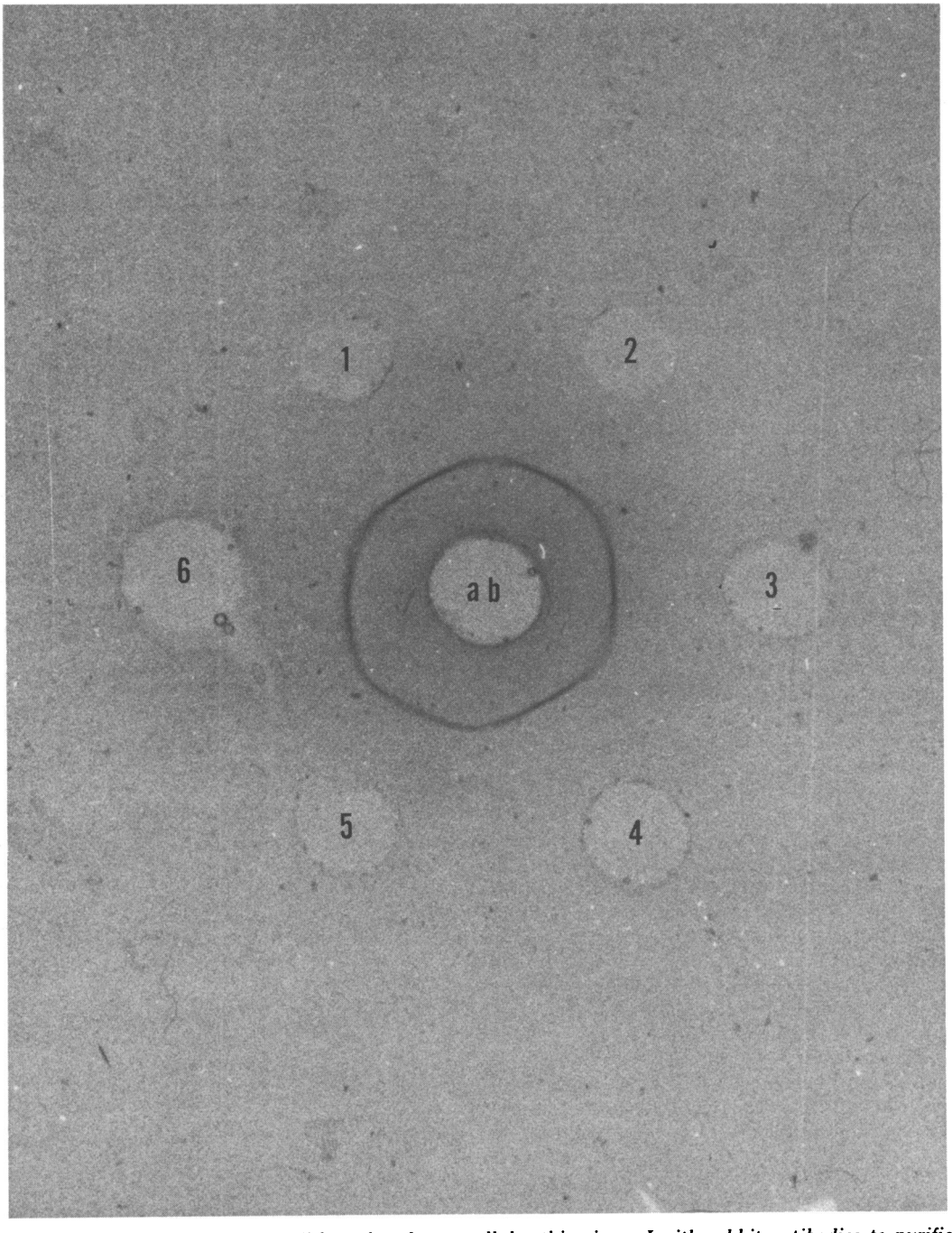

FIG. 5. *Reaction of purified cell-bound and extracellular thiaminase I with rabbit antibodies to purified extracellular thiaminase I. (ab, antithiaminase I; 1, 2, 4-purified cell-bound thiaminase I; 3, 5, 6-purified extracellular thiaminase I).*

1.65. The observed specific activity of several of the preparations of purified cell-bound thiaminase I of 6,000 to 7,000 mU per mg of protein agree well with the 6,100 mU per mg of protein value previously reported by Wittliff and Airth (13) for the extracellular enzyme. Both cell-bound and extracellular thiaminase I demonstrate the same 44,000 to 45,000 dalton mass upon SDS-polyacrylamide electrophoresis gels and ultracentrifugation analysis. Lastly, by using the assay conditions of Wittliff and Airth, the K_m and V_{max} values determined for cell-bound thiaminase I are the same as those previously reported for extracellular thiaminase I (13).

DISCUSSION

Two lines of evidence suggest that the cell-bound enzyme is the immediate precursor for extracellular thiaminase I. First, extracellular thiaminase I is produced by washed cells at the expense of cell-bound enzyme (Tables 2 and 3, and Fig. 4). Secondly, thiaminase I released from the cell at low pH is indistinguishable by various physical and kinetic criteria from extracellular enzyme produced during the culture cycle.

The inability of certain inhibitors of protein synthesis, oxidative phosphorylation, or transcription or the absence of carbon sources, nitrogen sources, or divalent cations to affect the release of cell-bound thiaminase I implies that the release of the enzyme is independent of its synthesis. However, Fig. 2 indicates that the amount of cell-bound thiaminase I per cell reaches a plateau of about 1,000 molecules of enzyme per cell toward the end of the logarithmic phase of growth, whereas the amount of extracellular enzyme continues to rise. This suggests that under physiological conditions the synthesis and release of the enzyme may not be totally independent processes. Specifically, one can envision that when all of the binding sites for thiaminase I are occupied that the rate of synthesis influences the rate of release or vise versa. In addition, the effects of pH and osmotic pressure on the release of the enzyme suggest that cell-bound thiaminase I is located at or near the surface of the cell, perhaps within a mesosome or in the periplasmic space. Both of these two locations are subject to alteration by changes in pH or osmotic pressure.

Nothing is known about the physiological role of thiaminase I in the cell's metabolism, yet a comparison of the effect of pH on the catalytic activity of the enzyme in the base-exchange reaction to its effect on the release of the enzyme from the cell suggests that it is the cell-bound form of thiaminase I which is physiologically important to the organism. Thiaminase I exhibits maximum catalytic activity over a broad range of pH values from around 5.6 and 7.0. The catalytic activity falls off rather sharply in both the acidic and basic directions. Little or not activity remains below pH 4.2 or above pH 8.5 (13). Thus, full catalytic activity coincides with minimum release from the cell.

ACKNOWLEDGMENTS

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